Widespread transcriptional gene inactivation initiated by a repair intermediate of 8-oxoguanine

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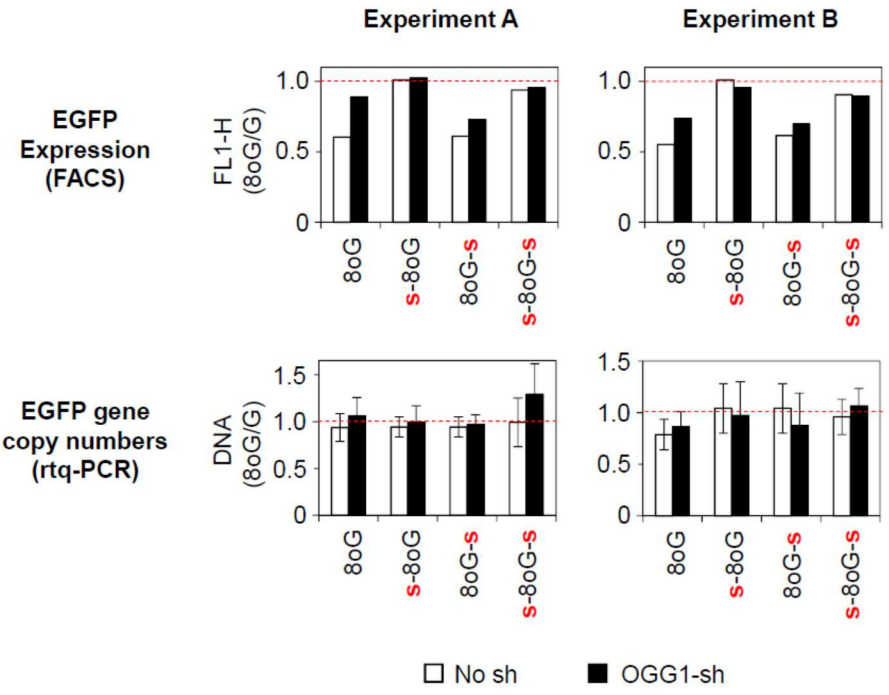
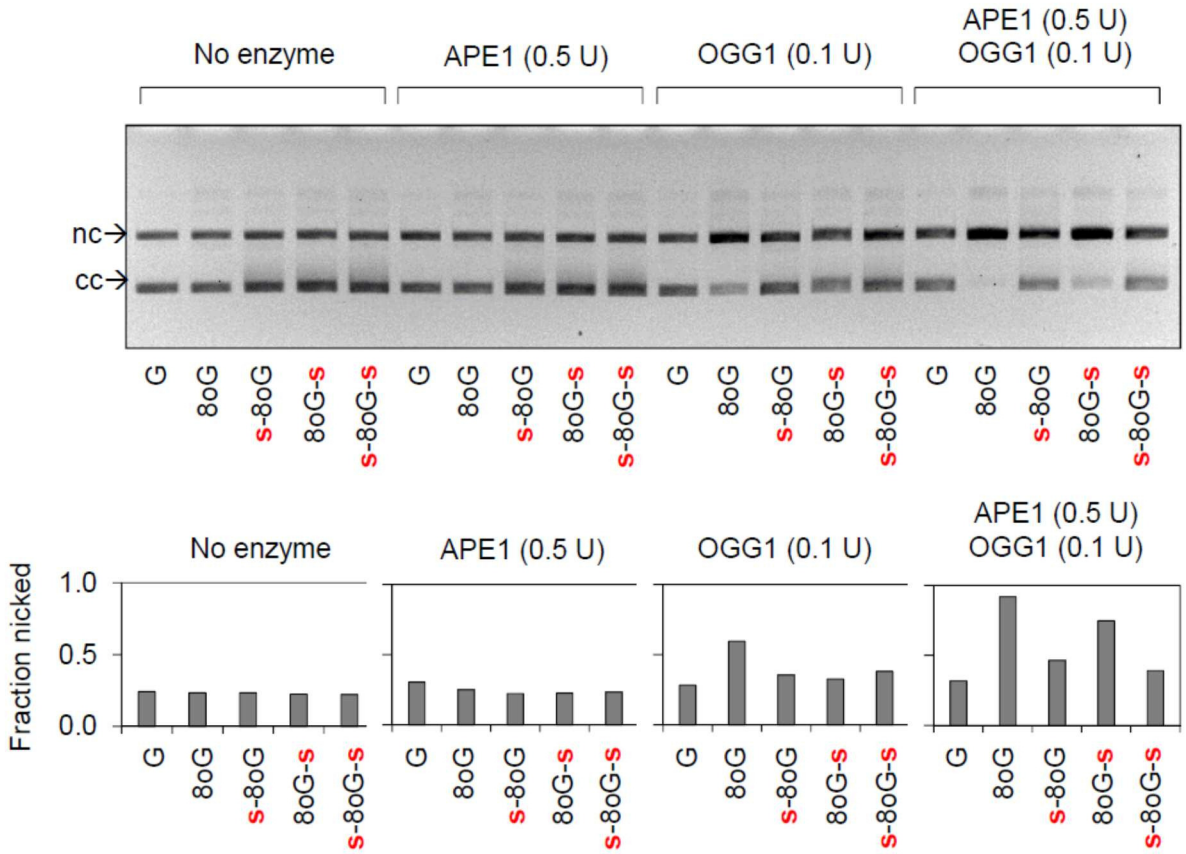
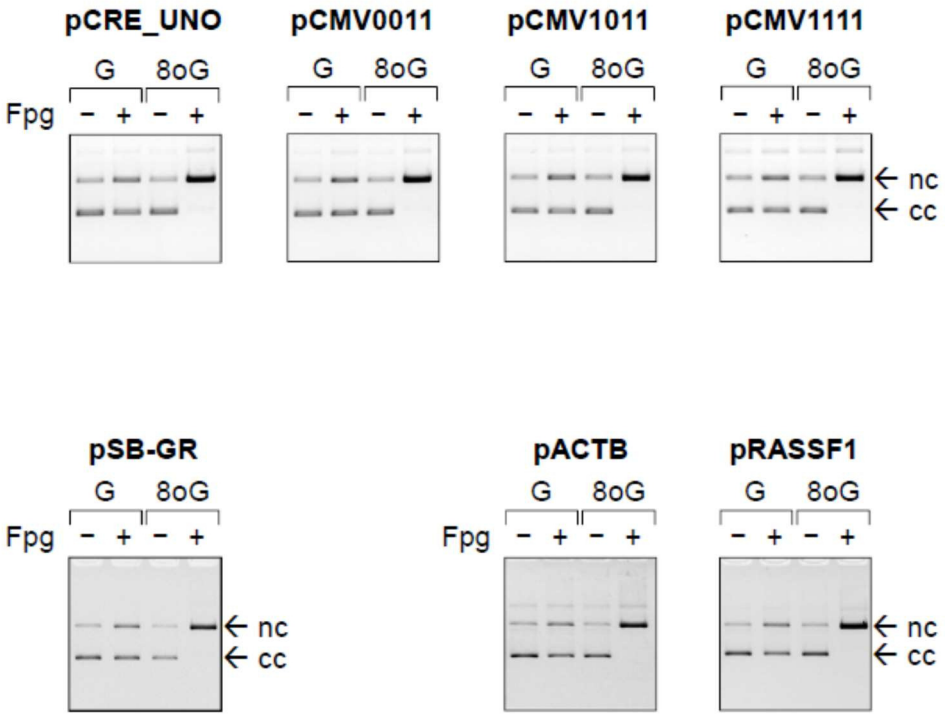
SUPPLEMENTARY DATA



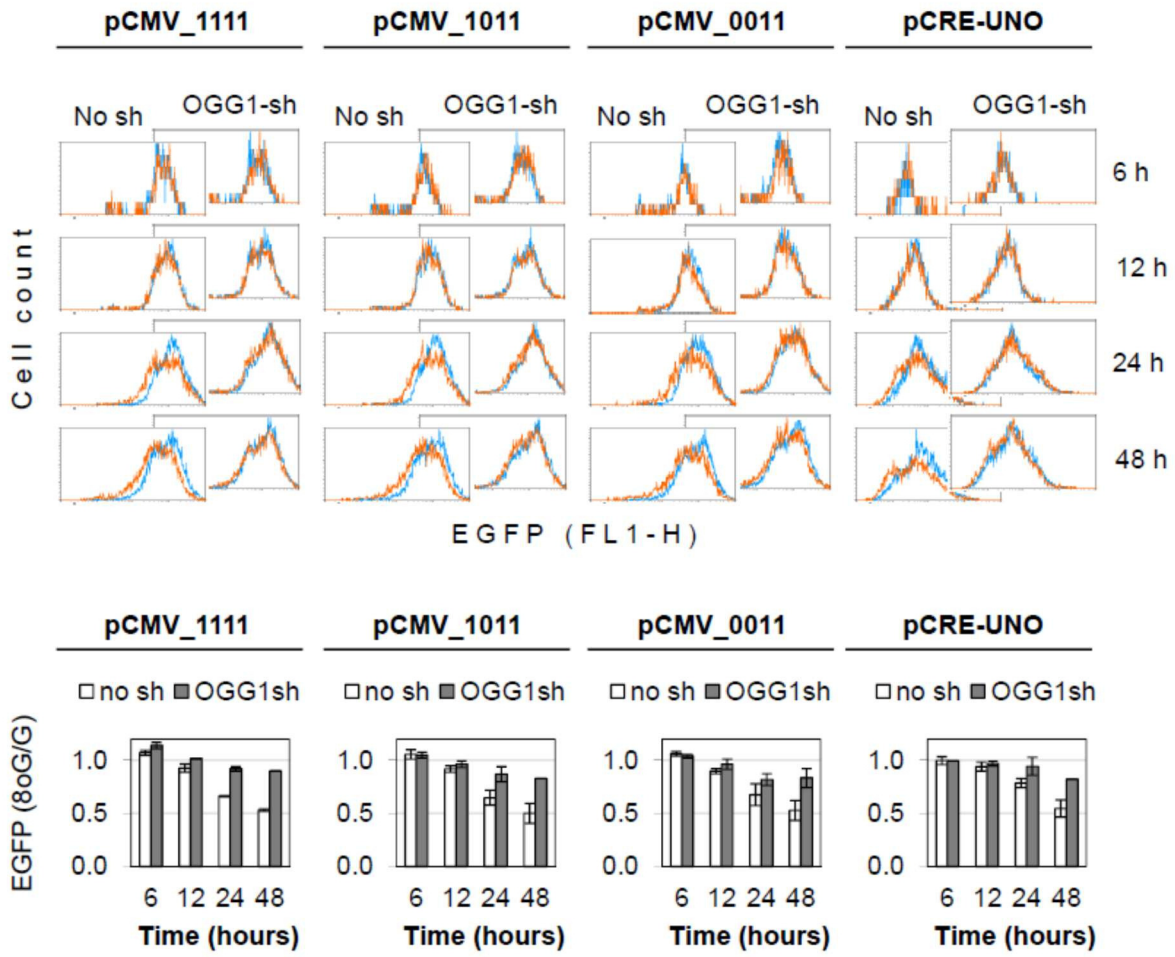
Supplementary Figure S1. Parallel quantification of EGFP protein expression (top panels) and the EGFP gene copy numbers (bottom panels) recovered from transfected cells 48 hours post transfection with the constructs containing the specified modifications, calculated (8oG/G) relative to the respective construct with incorporated unmodified nucleotide (mean±s.d.). Both types of analyses were performed in the same transfected cell samples, as explained in the Supplementary Methods section. Real time quantitative PCR analyses (rtq-PCR) were performed in quadruplicates and all single values normalised for the concomitantly determined copy numbers of the co-transfected unmodified vector as an internal reference.



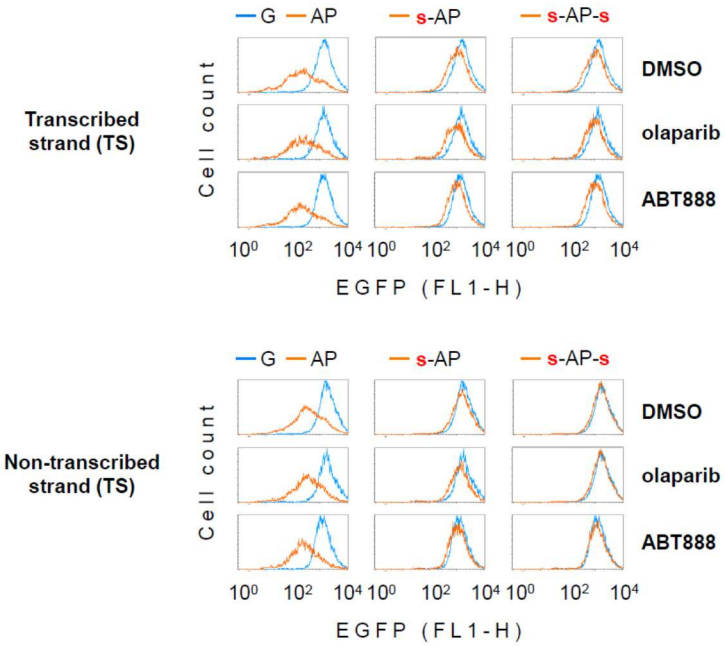
Supplementary Figure S2. DNA strand cleavage activity of human OGG1 in the presence of APE1 towards plasmid substrates containing the specified modifications. Agarose gels and quantification of the fraction of nicked DNA. The amounts of OGG1 per reaction were intentionally reduced from 0.5 (in Figure 1) to 0.1 units, in order to better appreciate the stimulatory effect of APE1.



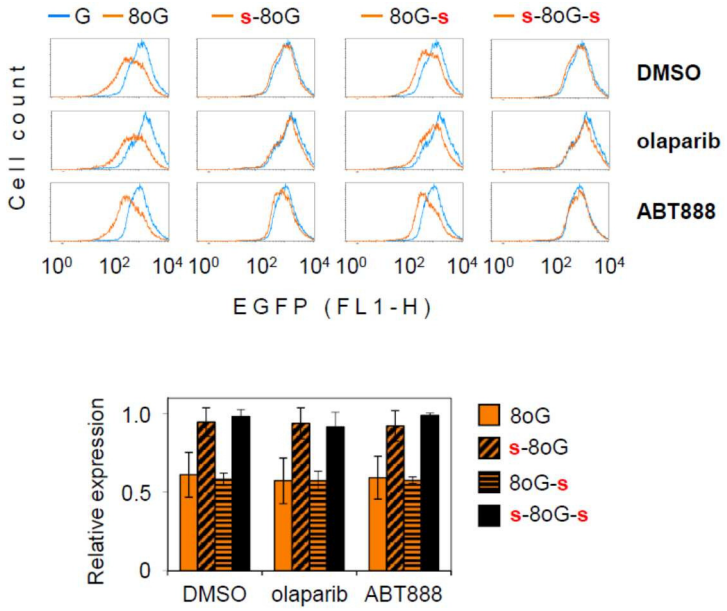
Supplementary Figure S3. Verification of 8-oxoG incorporation into the specified vectors by incubation with bacterial Fpg. DNA strand scission analysis of constructs produced with unmodified synthetic oligonucleotide (G) or the oligonucleotide containing single 8-oxoG (8oG) by conversion of covalently closed DNA (cc) to the nicked circular (nc) form.



Supplementary Figure S4. OGG1 knockdown attenuates the negative effect of 8-oxoG on expression driven by the specified truncated CMV promoter variants. Fluorescence distribution plots show EGFP expression in the OGG1 knockdown (OGG1-sh) HeLa cells and the isogenic control cell line (No sh) transfected with the specified constructs containing G (blue lines) or 8-oxoG (amber lines). Bar charts below show mean EGFP expression, calculated relative to the respective construct without 8-oxoG (n=2). Error bars indicate data range.



Supplementary Figure S5. Expression of constructs containing synthetic AP lesion (tetrahydrofuran) in the indicated strand of the EGFP gene in HeLa cells incubated in the absence (DMSO) and in the presence of the indicated PARP inhibitors. Some of the constructs contained phosphorothioate linkages next to the AP site, as indicated. Overlaid fluorescence distribution plots obtained in a representative experiment (24 hours post transfection). Summary of three independent experiments is presented in Figure 8D.



Supplementary Figure S6. Expression of constructs containing 8-oxodG and the indicated phosphorothioate linkages next to the modified nucleotide in HeLa cells incubated 24 hours in the absence (DMSO) and in the presence of the indicated PARP inhibitors. Overlaid fluorescence distribution plots obtained in a representative experiment (top) and summary of three independent experiments (bottom) (mean±s.d.).

SUPPLEMENTARY METHODS

Construction of expression vectors

Tetracycline-regulated (tet-on) promoter was constructed by insertion of two binding sites ($TetO_2$) for the tetracycline-controlled repressor protein (TetR) immediately downstream from the transcription start site by recombination in the SCS-8 E.coli strain (Agilent Technologies, Waldbronn, Germany) using the Gibson Assembly® Cloning Kit (NEB GmbH, Frankfurt am Main, Germany). The pZAJ-5C recombination template was generated by PCR (20 cycles) using Phusion HF DNA Polymerase (Thermo Fischer Scientific, Darmstadt, Germany). Primers were 5′-AGATCCGCTAGAGCAATG and 5′-CTGATAGGGATCACTAAACCAGCTCTGC. Template DNA was digested with DpnI (Thermo Fischer Scientific, Darmstadt, Germany) prior to the assembly reaction. The partner $TetO_2$ fragment was obtained by annealing oligonucleotides with the sequences 5′-TCCCTATCAGTGATAGAGATCTCCCTATCAGTGATAGAGATCGTCGAGATCCGCTAGAGCAATG and 5′-CGACGATCTCTATCACTGATAGGGAGATCTCTATCACTGATAGGGATCACTAAACCAGCTCTGC. Constructs containing the specified human promoters or the synthetic GR-TK promoter were generated by Gibson assembly, as described above; however pZAJ-5C vector was amplified without the CMV-IE promoter region with primers 5′-AGATCCGCTAGAGCAATG and 5′-CACAGAATCAGGGGATAACG. ACTB, RASSF1 and GR_SynthRE promoter sequences were obtained from the pLightSwitch clones (Active Motif; La Hulpe; Belgium) by PCR using the following primers: 5′-CGTTATCCCCTGATTCTGTGCCGGTACCTGAGCTCTTA and 5′-CATTGCTCTAGCGGATCTAAAGGCGAGGCTCTGAGA (ACTB); 5′-CGTTATCCCCTGATTCTGTGAGTCAGACCTTCCTGACT and 5′-CATTGCTCTAGCGGATCTCTCAGGCTCCCCCGACAT (RASSF1); 5′-CGTTATCCCCTGATTCTGTGAGTGCAGGTGCCAGAACATT and 5′-CATTGCTCTAGCGGATCTCTTAAGCGGGTCGCTGCAGG (GR_SynthRE). PCR reactions were performed as described above, except the ACTB promoter, which was amplified 16 cycles with PfuTurbo® polymerase (Agilent Technologies).

Deletions of the specified regions of the CMV-IE promoter in the pZAJ vector were accomplished by intramolecular homologous recombination of corresponding PCR products in the transformed SCS-8 E.coli cells, as described above but insert omitted. The resulting promoters were assigned a 4-digit binary code in which each of the four CRE sequences is marked by 1 (if retained) or 0 (if deleted). PCR primers contained a deliberately chosen 24-nucleotide 5′-overhang, containing a cAMP response elements (CRE) sequence. Forward primer was 5′-CATTGCGTGACGTCAGCGCATTGCCCTGGCATTATGCCCAGTACA. Reverse primers were: 5′-GCAATGCGCTGACGTCACGCAATGGCGTACTTGGCATATGATACACT (CMV_1111); 5′-GCAATGCGCTGACGTCACGCAATGAGTCCCTATTGGCGTTACTATGG (CMV_1011); 5′-GCAATGCGCTGACGTCACGCAATGATGAACTAATGACCCCGTAATTGA (CMV_0011). Primers for the generation of promoter consisting of a single CRE site (CRE-Uno) were 5′-CATTGCGTGACGTCAGCGCATTGCTGTACGGTGGGAGGTCTATATAA and 5′-GCAATGCGCTGACGTCACGCAATGATGAACTAATGACCCCGTAATTGA. PCR (16 cycles) was performed with PfuTurbo® Polymerase.

Quantification of EGFP gene copy numbers recovered from transfected cells

All transfections were performed by the mixtures of the EGFP reporter constructs (with incorporated synthetic oligonucleotides, as specified) and the pDsRed-Monomer-N1 vector. The latter did not contain any artificially introduced modifications and was used for two purposes: as a flow cytometry gating marker for transfected cells and as an internal reference for DNA copy number analyses. At 6 hours after transfections, cells were divided in three parts. One was fixed immediately for quantitative EGFP analyses by flow cytometry, as described in Material and Methods. Because 8-oxoG does not yet exhibit the inhibitory effect on the gene expression at this early time point, equal EGFP expression served as an indicator that equal amounts of constructs containing G and 8-oxoG were actually delivered to cells (30). The remaining two samples of transfected cells were re-plated on the 6-well plates and cultivated for additional 42 hours. One part was further processed as described above for the EGFP fluorescence analyses by flow cytometry, whereas the parallel cell sample was used for isolation of total DNA. Cells were incubated 3 h at 50°C in 0.5% sodium dodecyl sulphate supplemented with 100 mg/L Proteinase K. DNA was isolated by standard phenol/chlorophorm extraction procedures, precipitated with ethanol and dissolved in a small volume (typically 40 microL) of 10 mM Tris-HCl (pH 8.0). Samples were further diluted to equilibrate DNA concentrations, based on the A260 values measured by the NanoDrop 2000 microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific) and the plasmid copy numbers in DNA samples were determined by real-time quantitative PCR.

Real-time quantitative PCR Light Cycler 1.5 and FastStart DNA MasterPLUS SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) were used according to the manufacturer's instructions. Primer pairs specific to coding regions of the reporter genes were the following: 5′-TCAAGGAGTTCATGCAGTTC and 5′-GAAGGACAGCTTCATGTAGT (DsRed-Monomer) along with 5′-GACCACTACCAGCAGAACAC and 5′- GCCTGTGCTTCTGCTAGGAT (EGFP). The EGFP-specific primer pair was intentionally designed to encompass the position of the incorporated 8-oxoG (29). Primer pairs were controlled by melting curve analyses and agarose gel electrophoresis to produce a single specific PCR product. DsRed-Monomer and EGFP gene fragments were amplified in parallel using the same conditions (annealing T=65°C, fluorescence acquisition T=88°C) and copy numbers were measured using the respective strandard curves, which had linear regression coefficients ≥0.99. All measurements were done in quadruplicates. For each run, the values obtained for the EGFP gene fragment were normalised by the measured amount of the reference pDsRed plasmid in the respective DNA samples, in order to correct for potentially variable transfection efficiencies. Finally, the recovered fractions of the damaged pZA plasmid were calculated relative to the recovery of the EGFP construct without 8-oxoG.